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No evidence for *AT2R* gene derangement in human urinary tract anomalies

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No evidence for *AT2R* gene derangement in human urinary tract anomalies.

Background. It has been recently found that mice, especially males, with a disrupted angiotensin type 2 receptor (*AT2R*) gene, which is located on the X-chromosome, often have a range of congenital anomalies of the kidney and urinary tract (CAKUT), including renal hypoplasia, and that Caucasian male patients with ureteropelvic junction stenosis (UPJ) and multicystic dysplastic kidneys frequently have A-G transition in intron 1 of the *AT2R* gene. We have previously found that renal hypoplasia is remarkably predominant in Japanese boys.

Methods. We investigated sex ratios for the frequency of each CAKUT. The frequency of the A-G transition between the controls and 66 Japanese boys with CAKUT were compared. There was renal hypoplasia in 16, UPJ in 17, vesicoureteral in 20, and other anomalies in 13. We also investigated whether any mutations in *AT2R* genes were detectable in patients with renal hypoplasia.

Results. In contrast to mice with a disruption of the *AT2R* gene, the male-to-female ratios in human patients proved to be considerably variable: 16 for renal hypoplasia, 2.1 for UPJ, 0.8 for vesicoureteral, and 1.2 for others. The frequency of the A-G transition was not different between the control population and the patients with CAKUT [31 of 102 (30%) vs. 23 of 66 (35%), respectively]. A sequencing study disclosed no mutations in nine boys with renal hypoplasia.

Conclusions. These findings indicate that the *AT2R* gene may not play a major role in the development of renal hypoplasia and other CAKUT in humans, at least in the Japanese population.

Congenital anomalies of the kidney and urinary tract (CAKUT) are now the major cause of renal failure in childhood [1]. Etiologies of most of these anomalies have not been defined. Nephrogenesis is controlled by many genes that regulate growth by affecting cell survival, proliferation, differentiation, and morphogenesis [2]. Recent studies identified several genes that were implicated in the nephrogenesis and in which the derangement resulted in renal maldevelopment [2–4]. Several human multiorgan syndromes involving renal malformations have a defined genetic basis [5].

The angiotensin type 2 receptor (AT2R) is considered to be associated with the development of fetal organs, since AT2R is expressed abundantly in fetal tissues [6–8]. Embryological development of the kidney is known to proceed by interaction between the ureteral bud and metanephric blastema. Recently, Ichikawa et al have proposed the molecular basis for the classic ureteral bud theory [9, 10]. According to this theory, expression of the AT2R in undifferentiated mesenchymal cells should lead to their apoptosis, and abnormalities in AT2R expression hinder the interaction between the ureteric bud and metanephric blastema and hamper the normal development of the nephron and ureter, accompanied with the development of CAKUT. Recent experimental studies observed that derangement of the renin-angiotensin system during fetal life resulted in renal maldevelopment [11]. The AT2R was recently identified as being located on the X-chromosome both in humans and mice and has been sequenced [12]. In male mice, it is also found that a specific disruption of the *AT2R* gene is often accompanied by a wide spectrum of anomalies confined to the urinary tract, such as renal hypoplasia, multicystic dysplastic kidneys (MCDK), ureteropelvic junction stenosis (UPJ), ureterovesical junction stenosis (UVJ), and vesicoureteral reflux (VUR). These are very similar to CAKUT in humans [13].

Previous studies in humans observed two polymorphic sites in the *AT2R* gene [13, 14]. Nishimura screened for

Key words: angiotensin II, renal hypoplasia, ureteropelvic junction stenosis, multicystic dysplastic kidney, CAKUT, gene mutation, Japanese population.

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the *AT2R* gene in Caucasian American and German patients with UPJ and MCDK and observed no mutations [13], but he found that the patients had the A-G transition at intron 1 of the *AT2R* gene more frequently than the control population. They observed that the A-G transition within the lariat branchpoint motif of intron 1 of an *AT2R* gene was associated with abnormal splicing of pre-mRNA, producing mRNA with a shorter length in a smaller amount than the normal allele. They found characteristics common to the human patients and the mutant mice for the *AT2R* genes: the same spectrum of anomalies, predominance in males, frequent unilaterality without other structural organ anomalies, and incomplete penetrance.

Our previous ultrasound screening study in neonates observed that renal hypoplasia, congenitally small kidneys with a reduced number of functioning nephrons, is remarkably predominant in males, as high an incidence as 1 in 300 boys [15]. The male predominance of the anomaly in humans suggests that the anomaly may be an X-linked genetic disorder.

In the present study, we examined whether Japanese patients with renal hypoplasia and other CAKUT had any association with the two sites of *AT2R* gene polymorphism reported thus far and whether patients with renal hypoplasia had any other *AT2R* gene mutations.

METHODS

Children who visited our hospitals from April 1997 to January 1999 with a diagnosis of CAKUT were enrolled in the present study. Informed consent for analysis of the *AT2R* gene in the patients was obtained from the parents of 66 Japanese boys with CAKUT. Hypoplasia was found in 16, aplastic dysplasia in 5, MCDK in 3, UPJ in 17, UVJ in 5, and VUR without renal maldevelopment in 20. The study protocol was in accordance with the standards of the ethics committee at each center.

Renal hypoplasia was diagnosed when technetium 99m dimercaptosuccinate (Tc 99m DMSA) renal scintigraphy showed a small functioning kidney with generally diminished tracer uptake [15]. On ultrasound, these kidneys appeared to be only slightly small without any cysts or enhanced echogenicity on ultrasound. The hypoplastic kidneys were unilateral in 16 and bilateral in 1 with a normal serum creatinine. The unilateral kidneys had more than 20% of a total tracer uptake on Tc 99m DMSA. These hypoplastic kidneys possibly contained dysplastic tissues such as primitive ducts or metaplastic cartilage. However, the possibility seemed very low because a previous histologic study observed dysplastic tissues rarely in human hypoplastic kidneys and almost exclusively in severe forms [16]. Sixteen boys with unilateral renal hypoplasia had no anomalies in the contralateral kidney. All hypoplastic kidneys and 9 of the 16 contralateral normal kidneys were associated with VUR. VUR was diagnosed by voiding cystourethrography. Aplastic dysplasia was defined as a very small rudimentary renal tissue on ultrasound without any function on Tc 99m DMSA, and MCDK was defined as multiple large cysts on ultrasound without any function on Tc 99m DMSA. Ureteropelvic junction was diagnosed as having pelvocalyceal dilation on ultrasound and stenosis at the ureteropelvic junction on excretion urography or Tc 99m diethylenetriamine pentaacetic acid (Tc 99m DTPA) renal scintigraphy, and UVJ as showing both pelvic and ureteral dilation on ultrasound and stenosis at ureterovesical junction on excretion urography or Tc 99m DTPA renal scintigraphy. Questioning anamnesis revealed that none of the 69 boys except one had a positive family history for CAKUT. One boy with VUR had a cousin with the same anomaly.

Genomic DNA was extracted from peripheral blood of 102 healthy Japanese male volunteers and 65 boys with CAKUT and was collected in tubes containing ethylenediaminetetraacetic acid (EDTA). A buffy coat was aspirated after centrifugation of the blood at 1500 rpm for 10 minutes. Leukocytes were collected by centrifugation at 2500 rpm for five minutes after hemolysis with the addition of a four times-diluted, phosphate-buffered saline (PBS) at 20 times volume. Genomic DNA was purified using Sepa Gene (Sanko Junyaku Co., Tokyo, Japan), following the manufacturer's instructions.

Analysis of polymerase chain reaction-restriction fragment length polymorphism

The subjects were analyzed for the two polymorphisms thus far reported in *AT2R* genes [13, 14]. The polymorphism in *intron 1* was examined following the method of Nishimura et al [13]. Genomic DNA was suspended in 10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 8.0, and was amplified by polymerase chain reaction (PCR) in a 20 μL reaction mixture containing 20 mmol/L Tris-HCl (pH 8.0), 50 mmol/L KCl, 2 mmol/L $MgCl_2$, 0.2 mmol/L each of four deoxynucleotide triphosphates, 1 unit of DNA polymerase (AmpliTaq Gold; Perkin Elmer, Foster City, CA, USA), and 100 ng genomic DNA using 0.4 mmol/L each of a primer pair of 5′-GGAAAGTAGAACATAC ATTAAATG-3′ and 5′-CCTGTAAGAGAAACAGCA GCTAAAGAATT-3′. Thermocycling in a GeneAmp PCR System 2400 (Perkin-Elmer) consisted of 9 minutes of preincubation at 95°C, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 2 minutes, extension at 72°C for 2 minutes, and final extension of 10 minutes at 72°C. PCR products were digested with the restriction enzyme EcoR I (Roche Molecular Biochemicals, Tokyo, Japan). The A-G transition yielded the EcoR I digestion site, thus providing restriction fragment length polymorphism.

The polymorphism in a 3′ untranslated region of exon

Table 1. Primer pairs prepared for investigation of sequence of antiotensin type 2 receptor

Primer	Forward	Reverse
A	ATCCCAGAGGCTATTTACTAACCA	GCTTTGCGCAGTTTTTGTTGG
B	TGCAATCTCCAACCCTCCAG	AACCCTTGCCAGCTTTCCAGA
C	ATTTGAATGAGCTGTTATGATTGG	AAACACAGGAAAAACAGAAGTTCA
D	CTCCCAGTGGACAGACCAAACA	GAACCTCCTAATAAAGAGCAAAAC
E	GGATGTCCTCAGCTCTGTATGTGT	TAGAGGAAGAGTAGCCAAAAGGAG
F	TTCTGGTCAATATTGTCGTGGTTA	CCATTGGGCATATTTCTCAGGT
G	TAGTTCCCCTTGTTTGGTGTATGG	CAAGAGGATGGCAAAAGGAAGT
H	TGCTCTGGCCTGGATGGGTGTC	TCTGGTGAGCCTCAAAGCAAGTAG

3 was examined following the previous report [14]. Genomic DNA was amplified by PCR in a similar way as described previously in this article, using 0.2 mmol/L each of a primer pair of 5′-GGATTCAGATTTCTCTTTGAA-3′ and 5′-GCATAGGAGTATGATTTAATC-3′. Thermocycling consisted of 9 minutes of preincubation at 95°C, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 50°C for 2 minutes, extension at 72°C for 2 minutes, and a final extension of 10 minutes at 72°C. PCR products were digested with restriction enzyme Alu I (GIBCO BRL) following the manufacturer's instructions. *C-A transition* lost the Alu I digestion site, thus yielding restriction fragment length polymorphism.

Sequence of AT2R

Genomic DNA from nine patients with renal hypoplasia and three normal male subjects was analyzed for the sequence of their *AT2R* genes. We prepared two sets of four pairs of primers (A to D and E to H; Table 1) to investigate the gene between nucleotides −2402 and −1147 from the translation initiation site and −88 and 1148, respectively (Fig. 1). PCR-amplified products using Pwo DNA Polymerase (Roche Molcecular Biochemicals) were purified with Wizard PCR Preps DNA Purification System (Promega) and directly sequenced with an ABI Prism 377 DNA Sequencer and Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Corp.).

Statistical analysis

The results were analyzed with StatView J-5.0 software. The statistical methods used were the chi-square test and the Fisher's exact probability test. A two-tailed *P* value of less than 0.05 was taken as the level of significance.

RESULTS

A total of 153 Japanese children visited our hospitals with a diagnosis of renal hypoplasia in 17 (16 boys and 1 girl), aplastic dysplasia in 10 (5 boys and 5 girls), MCDK in 8 (4 boys and 4 girls), UPJ in 22 (15 boys and 7 girls), UVJ in 8 (5 boys and 3 girls), and VUR in 88 (38 boys and 50 girls). The male-to-female ratio was 16 in renal hypoplasia, 1.0 in aplastic dysplasia, 1.0 in MCDK, 2.1 in UPJ, 1.7 in UVJ, and 0.8 in VUR.

Analysis of PCR-restriction fragment length polymorphism demonstrated that the frequency of A and G alleles at the A-G transition site (position −1332 from the translation initiation site) of intron 1 was 71 (70%) and 31 (30%), respectively, among the general male Japanese population. Boys with CAKUT showed no significant difference in regard to frequency of the G alleles in comparison with the general population: 23 (35%) among a total of 66 patients. These patients were categorized specifically as 6 of 16 (38%) in renal hypoplasia, 7 of 17 (41%) in UPJ, 1 of 5 (20%) in UVJ, 1 of 5 (20%) in aplastic dysplasia, 2 of 3 (67%) in MCDK, and 6 of 20 (30%) in VUR without renal maldevelopment, as shown in Table 2. There was no significant difference in the frequency of the G allele even when only severe cases of UPJ and VUR were considered: two of five boys (40%) with UPJ who needed surgical correction and 4 of 14 (29%) boys with VUR of grades 3 or more.

C and A alleles at the C-A transition site (position 1593) of exon 3 were completely linked to A and G alleles at the A-G transition site, respectively, in both the general population and patients with CAKUT (Table 2). Thus, there was no disequilibrium in frequency of the two alleles at exon 3 between the general population and the children with renal anomalies.

Sequencing of the *AT2R* gene revealed no mutation in the nine patients with renal hypoplasia, whereas it identified a polymorphism (A/T) at position −1189 of the intron 2 (Fig. 1). A sequencing study using D primer pairs in 18 control subjects and another 35 patients with CAKUT confirmed the existence of A/T polymorphism. The T allele at the A-T transition site was found in only subjects with G allele at the A-G transition site in intron 1, being found in 7 of 22 patients and 5 of 11 control subjects. The results of analysis of PCR-restriction fragment length polymorphism in intron 1 agreed completely with the results of sequencing in all of the 62 subjects examined.

DISCUSSION

Analysis of PCR-restriction fragment length polymorphism showed that the A-G transition in intron 1 of the *AT2R* gene was also found in the Japanese population

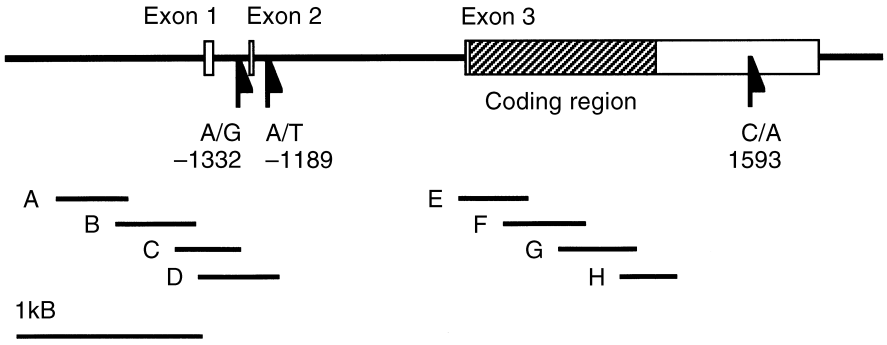

Fig. 1. An illustration of the human *AT2R* gene and localization of three polymorphic sites (A/G, A/T, and C/A) and primer pairs (A–H) used for the sequencing study.

Table 2. Frequency of the A/G and C/A alleles in polymorphic sites of intron 2 and exon 3, respectively, in the general Japanese male population and boys with congenital anomalies of the kidney and urinary tract

	Polymorphic site of intron 1		Polymorphic site of exon 3	
Subjects	A	G	C	A
General population	71	31 (30%)	28	12 (30%)
Hypoplasia	10	6 (38%)	10	6 (38%)
Aplastic dysplasia	4	1 (20%)	4	1 (20%)
MCDK	1	2 (67%)	1	2 (67%)
UPJ	10	7 (41%)	10	7 (41%)
UVJ	4	1 (20%)	4	1 (20%)
VUR	14	6 (30%)	14	6 (30%)

There was no significant difference in frequency of each allele between the general population and patients with each anomaly. Abbreviations are: MCDK, multicystic dysplastic kidneys; UPJ, ureteropelvic junction stenosis; UVJ, ureterovesical junction stenosis; VUR, vesicoureteral reflux.

with a similar frequency to that of the Caucasian American and German populations and that the incidence of the A-G transition was not different between the general Japanese population and Japanese patients with CAKUT (including renal hypoplasia, UPJ, and VUR). Sequencing of the intron 1 of the *AT2R* gene confirmed these results. PCR-restriction fragment length analysis of C/A polymorphism in exon 3 of the *AT2R* gene showed that the C-A transition in exon 3 is linked to the A-G transition in intron 1 and that there was no difference in the incidence of C-A transition between patients with CAKUT and the general population. The present study found a new A/T polymorphism in intron 2 of the *AT2R* gene. The incidence of A-T transition did not differ between the general population and patients with renal hypoplasia and other CAKUT. Nishimura observed the A-G transition in intron 1 of the *AT2R* gene more frequently in Caucasian American and German patients with UPJ and MCDK than in the general population. The present study found no significant difference in the incidence of the A-G transition between the general Japanese population and patients with CAKUT such as renal hypoplasia, UPJ, and VUR. The present study involved too few patients with UVJ, aplastic dysplasia, and MCDK for statistical analysis. The discrepancy between the two studies may be explained by the difference in genetic backgrounds, since Nishimura was able to increase the penetrance of the CAKUT in mice with *AT2R* gene disruption by interbreeding alone, indicating the existence of a modifier gene.

Nishimura observed that male preponderance was the common characteristic for CAKUT in both mice with *AT2R* gene disruption and human patients [13]. However, male preponderance has been observed in only UPJ, UVJ and renal agenesis, and not in MCDK and VUR [17]. VUR in humans is well explained as being inherited in an autosomal dominant manner [18]. Recently, Feather et al revealed in their linkage analysis study that several autosomal chromosomes, excluding the X-chromosome, showed positive linkage to VUR with chromosome 1 showing the most positive [19]. Groenen et al reported a fetus with multicystic kidneys with a translocation disrupting the *CDC5 l* gene on chromosome 6 and another cell cycle gene on chromosome 19, suggesting a potentially responsible gene locus on these chromosomes [20]. In our previous study, a striking male preponderance was found in renal hypoplasia with a high incidence of 1 in 300 boys [15]. Thus far, there have been no other reports that demonstrate such a high incidence of renal hypoplasia in boys. This is understandable because of the evidence (*1*) that it is very hard with ultrasound to detect hypoplastic kidneys early in life, since the hypoplastic kidneys even with such a poor function (from a third to a half of the function in the contralateral normal kidneys) are only very slightly smaller in longitudinal length without any abnormal echogenicity or abnormal structures on ultrasound [15], and (*2*) that patients with renal hypoplasia are usually identified only after they develop urinary infection, when their small kidneys are often diagnosed as reflux nephropathy (postnatally acquired atrophic kidneys) because of the concomitant VUR. In the present multicenter study, a remarkable male preponderance was also found. These findings indicate that the male preponderance differs considerably according to specific human CAKUT. Since mice with a disruption of the *AT2R* gene, which is located on the X-chromosome, had a range of CAKUT with a simi-

lar male preponderance, other major causes should exist in human MCDK and VUR that do not show male preponderance.

Nishimura identified no mutations other than frequent A-G transition in intron 1 of the *AT2R* gene in patients with UPJ, which was predominant in males [13]. The A-G transition does not play a decisive role by itself in the development of CAKUT, since approximately 40% of the Caucasian control populations also had the A-G transition. The present study identified no mutations in the *AT2R* gene of patients with renal hypoplasia. These findings indicate that *AT2R* gene derangement may not play a major role in the development of either CAKUT, such as renal hypoplasia, and UPJ, which show male preponderance.

In the present study, familial occurrence was almost nonexistent except for one family with VUR. CAKUT is usually unilateral without any symptoms and often may not be diagnosed in humans throughout an entire lifetime. Voiding dysfunction is known to play a major role in increasing the grade of VUR and developing symptomatic urinary infection in patients with VUR [21–23]. Most of the VUR cases that were diagnosed on screening for family members of the index patient with VUR were low grades without voiding dysfunction [21, 24] and could be expected to be asymptomatic and recover spontaneously [25]. These clinical features allow human familial CAKUT to be assumed more rarely than they actually are diagnosed. Nishimura demonstrated that CAKUT in mice with an *AT2R* disruption were inherited with 21% penetrance in males and with 5% in females [13]. They ascribed the incomplete penetrance to the existence of other modifier genes and environmental factors. Only in the VUR, UPJ, bilateral renal agenesis, and renal dysplasia among CAKUT in humans was familial occurrence identified [26–29]. Familial occurrence was commonly found for VUR with the incidence of 20% [30], whereas it appeared only rarely for UPJ, bilateral renal agenesis, and renal dysplasia [26–28]. Frequency of familial occurrence varied among specific CAKUT in humans, in contrast to that in mice with *AT2R* gene disruption. The discrepancy suggests that some factors other than the *AT2R* gene may play a major role in the development of human CAKUT. A great deal of genes have been nominated as a cause of human CAKUT with the same phenotype [29]. More investigations should be carried out for unraveling the etiology of specific human CAKUT.

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